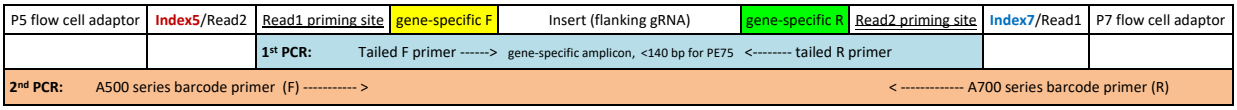
Tsai Lab Amplicon Sequencing Protocol v2.2

Adopted from Jacobs et al. (2015) BMC Biotechnology 15: 16.
Zhou X et al. (2015) New Phytologist 208: 298-301

Perform two PCR reactions to incorporate first Illumina sequencing primers and then barcodes/flow cell adaptors.

Your final amplicon will look like this:



Relevant sequences are:

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5'AATGATACGGCGACCACCGAGATCTACAC-INDEXi5-ACACTCTTTCCCTACACGACGCTCTTCCGATCT[GS-F primer] 3'
5'     CAAGCAGAAGACGGCATACGAGAT-INDEXi7-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC[GS-R primer] 3'
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Design your gene-specific (GS) primers with an amplicon length appropriate for the preferred sequencing platform (we usually target 110-130 bp for NextSeq PE75 or 250-290 bp for MiSeq PE150).

Include wobbles as necessary for SNPs or for amplification of closely related genes. We keep the oligo length below 50 mers, as longer oligos require a higher synthesis scale (more expensive) with Sigma.

Example of tailed primers:

```
4CL15(723F)tailF      CCTACACGACGCTCTTCCGATCTGACAAYCCTAACCTRTATTTTCACAG
4CL15(891R)tailR      GTTCAGACGTGTGCTCTTCCGATCTTCTCAAYCAATCCCAGCAAAG
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Index primers:

```
A500 series    AATGATACGGCGACCACCGAGATCTACAC[NNNNNNNN]ACACTCTTTCCCTACACGACGCTCTTCCGATCT
A700 series    CAAGCAGAAGACGGCATACGAGAT[NNNNNNNN]GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC
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A5 Index	Sequence	Rev. complement	A7 Index	Sequence
A501	TGAACCTT	AAGGTTCA	A701	ATCACGAC
A502	TGCTAAGT	ACTTAGCA	A702	ACAGTGGT
A503	TGTTCTCT	AGAGAACA	A703	CAGATCCA
A504	TAAGACAC	GTGTCTTA	A704	ACAAACGG
A505	CTAATCGA	TCGATTAG	A705	ACCCAGCA
A506	CTAGAACA	TGTTCTAG	A706	AACCCCTC
A507	TAAGTTCC	GGAACTTA	A707	CCCAACCT
A508	TAGACCTA	TAGGTCTA	A708	CACCACAC
During sequencing, A5 index sequence is obtained in Read2 in reverse complement. Hence, you need to enter both the original and reverse complemented sequences for A5's on the sample submission sheet. **Tsai lab members: use the NA500 & NA700 primers.**			A709	GAAACCCA
			A710	TGTGACCA
			A711	AGGGTCAA
			A712	AGGAGTGG

A total of 8x12 = 96 barcode combinations are available. Barcode by transgenic plant line, *i.e.*, each gDNA will be assigned the same barcode regardless of the amplicons. For example, in the Zhou et al. (2015) New Phytologist study, we edited two genes, *4CL1* and *4CL2*, in separate transformation trials. The *4CL1* gRNA is not expected to target its paralog *4CL5*. We designed a pair of (tailed) primers that amplify both *4CL1* and *4CL5*, and another pair for *4CL2*. Two PCR reactions were done (*4CL15* and *4CL2*) for each transgenic line, and attached to the same A5/A7 barcode combination. The three amplicon (gene) sequences can be resolved bioinformatically during read mapping. So in total, you can screen up to 96 plants with many amplicons per plant. When pooling different amplicons, it is useful to design them with different lengths for ease of tracking during PCR QC.

Amplicon library preparation

Isolate genomic DNA following the "Plant Genomic DNA Extraction miniprep" protocol. gDNA is resuspended in RNase-containing ddH_2O, so NanoDrop analysis is NOT needed (useless).

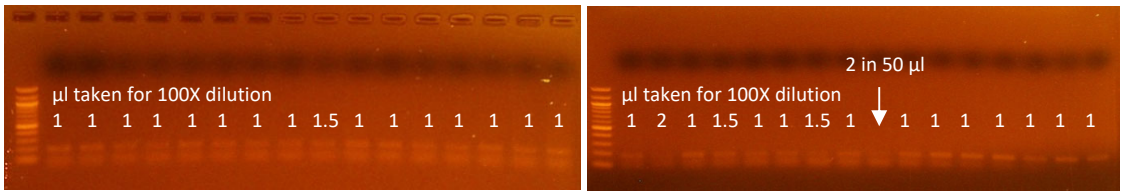
Gel-QC to assess gDNA quality and evaluate concentration based on MW markers.

Because the extension step is short with fewer cycles than standard PCR (intentional to avoid over-amplification), because data coverage is deep (100's and 1000's of reads), and because the predominant CRISPR mutations are indels, standard Taq works as well as high fidelity Taq in our hands.

1. First PCR: **always prepare a mater mix, with 10% extra**

gDNA (1 ng)	1 µl	94°C x 3 min
TailF primer (3 µM)	1 µl	[94°C x 30 sec
TailR primer (3 µM)	1 µl	55°C x 45 sec (or 2°C below primer Tm)
GoTaq 2X master mix	5 µl	72°C x 20 sec] (60 sec/kb)
ddH_2O	to 10 µl	25 cycles, 72°C x 2 min, hold at 25°C

2. Check 4 µl on a gel to confirm amplification. Faint bands are fine.

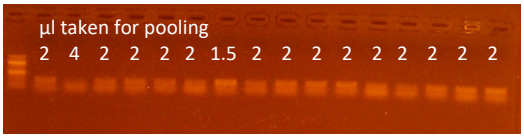


3. Dilute and pool PCR products/amplicons by plant line (*e.g.*, combine 4CL15 and 4CL2 amplicons from the same plant). Take 1 µl if the band is strong, 2 µl if weak, and 3-4 µl if barely visible (see examples above). This part is not terribly accurate, but it works fine. The PCR products are then diluted into 100 µl of water.

4. Second PCR using the **NA500** and **NA700** index primers: Set up multiple reactions, one per plant, each with a different index primer combination. The same index primer combination can be used for different amplicons, if necessary. Plan ahead in an Excel spreadsheet to help with tracking!

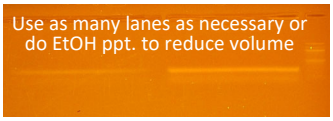
Diluted 1st PCR pool	1 µl	94°C x 3 min
A5 F primer (3 µM)	1 µl	[94°C x 30 sec
A7 R primer (3 µM)	1 µl	55°C x 45 sec (or 2°C below primer Tm)
GoTaq 2X master mix	5 µl	72°C x 20 sec] (60 sec/kb)
ddH_2O	to 10 µl	10 cycles, 72°C x 2 min, hold at 25°C

5. Check 4 µl on a gel to confirm amplification. There should be a band for each amplicon in the pool. Also check for cross contamination. See the examples on the right – substantially more primer dimers (than 1st PCR above), because of the intentionally low amplification cycles.



6. Mix the remaining PCR products together (everything is indexed), adjust the amount according to intensities (see notes above). If necessary, reduce sample volume to ~20 µl by ethanol precipitation.

7. Load everything in a **1.5%** TAE agarose + 1 mM cytidine. Add 1 mM cytidine to the running buffer, and run the gel at 25V (see gel to the right). Cut out the correct range, gel-purify and perform Qubit quantification.



8. UGA sample submission requires ~6 nM x 20 µl for sequencing. When amplicon sizes are similar, they appear as one band as shown on the right.